

# Expression of type I GNRH receptor and *in vivo* and *in vitro* GNRH-I effects in corpora lutea of pseudopregnant rabbits

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## Abstract

The expression of type I GNRH receptor (GNRHR-I) and the direct role of GNRH-I on corpora lutea (CL) function were studied in the pseudopregnant rabbit model. Immunohistochemistry evidenced GNRHR-I and GNRH-I in luteal cells at early (day 4 pseudopregnancy)-, mid (day 9)-, and late (day 13)-luteal stages. Real-time RT-PCR and western blotting revealed *GNRHR-I* mRNA and protein at the three luteal stages. Buserelin *in vivo* treatment at days 9 and 13 decreased plasma progesterone levels for 48 and 24 h respectively. In *in vitro* cultured CL, buserelin reduced progesterone secretion, increased prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) secretion and cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS) activities at days 9 and 13, and decreased  $PGE_2$  at day 13. Co-incubation with antagonists for GNRH-I (antide), inositol 1,4,5-trisphosphate ( $IP_3$ , 2-amino-ethoxydiphenylborate), and

diacylglycerol (DAG, 1-hexadecyl-2-acetyl glycerol) or inhibitors for phospholipase C (PLC, compound 48/80), and protein kinase C (PKC, staurosporine) counteracted the buserelin effects. Buserelin co-incubated with COX inhibitor (acetylsalicylic acid) increased progesterone and decreased  $PGF_{2\alpha}$  and NOS activity at days 9 and 13, whereas co-incubation with NOS inhibitor (*N*-nitro-L-arginine methyl ester) increased progesterone at the same luteal stages. These results suggest that GNRHR-I is constitutively expressed in rabbit CL independently of luteal stage, whereas GNRH-I down-regulates directly CL progesterone production via  $PGF_{2\alpha}$  at mid- and late-luteal stages of pseudopregnancy, utilizing its cognate type I receptor with a post-receptorial mechanism that involves PLC,  $IP_3$ , DAG, PKC, COX-2, and NOS.

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## Introduction

GNRH is a hypothalamic neuronal secretory decapeptide that plays a central regulatory role in mammalian reproductive physiology. GNRH influences reproductive processes mainly by regulating pituitary gonadotropin synthesis and release, which in turn modulate steroidogenesis and gametogenesis (Conn & Crowley 1994). Even if the hypothalamus and pituitary are, respectively, the principal source of and target site for GNRH, several studies have reported an extra-hypothalamic origin of GNRH, as well as an extrapituitary presence of GNRH receptor (GNRHR) in numerous peripheral tissues including reproductive organs, such as testis, prostate, ovary, oviduct, placenta, and mammary gland (Ramakrishnappa *et al.* 2005).

There are currently 14 identified naturally occurring GNRH structural variants across the vertebrate species (Somoza *et al.* 2002). However, it is evident that at least three forms of GNRH are present in the majority of the vertebrate species studied: hypothalamic GNRH (GNRH-I),

mid-brain GNRH (GNRH-II), and telencephalic GNRH (GNRH-III) (Gorbman & Sower 2003). In extrapituitary reproductive tissues, GNRH is considered to act in an autocrine or a paracrine manner and to regulate ovarian steroidogenesis by exerting a stimulatory as well as an inhibitory effect on the production of steroid hormones and apoptosis in ovarian follicles and corpora lutea (CL; Dubois *et al.* 2002, Ramakrishnappa *et al.* 2005). Although three types of GNRHR have been identified thus far, mammals have only two types of receptors: GNRHR-I and -II (Millar 2005). All known GNRHRs are transmembrane G protein-coupled receptors that produce their effects by activating phospholipase A2 (PLA2), phospholipase C (PLC), PLD, or adenylate cyclase (AC) cell signaling pathways (Millar 2005). Phospholipase activation may generate arachidonic acid (AA) that is converted into prostaglandins (PGs) by the action of the cyclooxygenase-1 and -2 (COX-1 and -2) as well as by that of other PG synthase enzyme types (Naor 2009).

Various studies have shown that GNRH exerts both inhibitory and stimulatory effects in the gonads, with either

down- or up-regulation of the ovarian cellular steroid production (Ramakrishnappa *et al.* 2005). Few studies have addressed the direct effects of GNRH in rabbit CL, with conflicting results: earlier works pointed to CL regression with serum progesterone decline (Hilliard *et al.* 1976, Rippel *et al.* 1976), while a later study indicated that there was no effect on steroid production by ovary cultured *in vitro* (Eisenberg *et al.* 1984). Therefore, the main objectives of the present study were to examine the *in vivo* and *in vitro* modulatory effects of GNRH on rabbit CL during early-, mid-, and late-luteal stages of pseudopregnancy. With this end in view, experiments were devised to clarify the presence of GNRHR-I and GNRH-I and their possible post-receptorial mechanisms, as well as the effects of GNRH-I on the production of progesterone, PGE<sub>2</sub>, and PGF<sub>2α</sub> and on the activities of COX-1 and -2 and nitric oxide synthase (NOS), the enzymes involved in the regulation of rabbit CL life span (Boiti *et al.* 2000, 2005, Zerani *et al.* 2007).

## Materials and Methods

### Reagents

Reagent for isolation of total RNA (TRIzol) was purchased from Invitrogen. iSCRIPT cDNA and iQ SYBR Green SuperMix were purchased from Bio-Rad. QIAquick PCR Purification Kit for sequencing PCR product was from Qiagen. Random hexamer primers, DNase I Amplification Grade, real-time PCR primers for *GNRHR-I* and *18S* were supplied by Invitrogen.

Tritiated progesterone, PGF<sub>2α</sub>, PGE<sub>2</sub>, and AA were purchased from Amersham Biosciences Ltd, while non-radioactive hormones and specific antisera were from Sigma-Aldrich. The NOS detect TM assay kit was purchased from Alexis Corp. (Läufelfingen, Germany).

Goat polyclonal anti-GNRHR-I primary antibody (sc-8681) and biotin rabbit anti-goat IgG secondary antibody (sc-2774) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti GNRH-I primary antibody (MAB 5456) was purchased by Chemicon International (Temecula, CA, USA) and biotin goat anti-mouse IgG secondary antibody was obtained by Zymed Laboratories (South San Francisco, CA, USA). The avidin-biotin complex (ABC, Vector Elite Kit) and the chromogen 3,3'-diaminobenzidine tetrachloride (DAB) were purchased from Vector Laboratories (Burlingame, CA, USA). The mouse monoclonal anti-β-tubulin antibody was from Sigma-Aldrich. PageRuler Ladder for western blot (WB) analysis was obtained from Fermentas (Burlington, Ontario, Canada). The enhanced chemiluminescence detection system for WB (Immobilon Western Chemiluminescent HRP Substrate) was purchased from Millipore (Billerica, MA, USA). The HRP-conjugated rabbit anti-goat IgG antibody as well as the Restore Western Blot Stripping Buffer was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

The Protran nitrocellulose membranes were from Whatman (Dassel, Germany). Biomax film for chemiluminescent blot imaging was from Kodak Laboratories. The bands were quantified using Quantity One software (Bio-Rad Laboratories).

The kit for the protein assay was purchased from Bio-Rad Laboratories. Medium 199 and Earle's Balanced Salt Solution were from Gibco. HEPES, BSA, buserelin, antide, eCG, 4-bromophenacyl bromide, compound 48/80, propranolol, 2-O-methyladenosine, acetylsalicylic acid (ASA), and N-nitro-L-arginine methyl were purchased from Sigma, 2-amino-ethoxydiphenylborate was from Tocris Cookson Ltd (Northpoint Fourth Way Avonmouth, UK), and 1-hexadecyl-2-acetyl glycerol was from Biomol Research Laboratories (Plymouth Meeting, PA, USA), whereas all other pure grade chemicals and reagents were obtained locally.

### Animals and hormonal regimen

Sexually mature New Zealand White female rabbits (3.5–4 kg body weight and 5 months of age) raised in premises owned by the University of Perugia were used for all experiments. The rabbits were housed individually in wire mesh cages under controlled light (14 h light:10 h darkness; lights off at 2100 h) and temperature (18–24 °C) conditions. Each animal had free access to food and water. Pseudopregnancy was induced with 20 IU eCG followed by 0.8 µg buserelin 3 days later (Stradaioli *et al.* 1997). The day of buserelin injection was designated as day 0. The animals (*n* = 5 for each luteal stage) were killed by cervical dislocation in accordance with the guidelines and principles for the care and use of research animals. The protocols involving the use of the animals for these experiments were approved by the Bioethic Committee of the University of Perugia.

### Tissue collection

Upon killing, reproductive tracts were promptly removed and thoroughly washed with saline. Within a few minutes, the CL were excised from ovaries and, after careful dissection of non-luteal tissue by fine forceps under stereoscopic magnification, immediately processed for *in vitro* experiments or frozen at –80 °C, after rinsing with RNase-free PBS, for later evaluation of gene and protein expression. For the immunohistochemical detection of GNRHR-I and GNRH-I, the ovaries of two additional animals for each luteal stage were fixed by immersion in 4% (w/v) formaldehyde in PBS (pH 7.4) for 24 h at room temperature, and subsequently processed for embedding in paraffin following routine tissue preparation procedures. The pituitary gland of a control rabbit was taken and processed as described above.

### Immunohistochemistry

Tissue sections were deparaffinized in xylene, rehydrated through graded ethanol, and finally rinsed in distilled water; then they were submitted to antigen retrieval by

microwaving in citrate buffer solution (10 mmol/l, pH 6) at 700 W for 10 min. The slides were cooled at room temperature. After rinsing with Tris-buffered saline (TBS), the specimens were dipped in 3% H<sub>2</sub>O<sub>2</sub> (v/v) in methanol for 1 h to quench the endogenous peroxidase activity and rinsed in TBS. Background labeling was prevented by incubating the sections with normal rabbit serum (for GNRHR-I) and normal goat serum (for GNRH-I), diluted 1:10, for 30 min at room temperature. Subsequently, the sections were incubated for 1 h at 37 °C and then overnight at 4 °C in a moist chamber with goat polyclonal anti-GNRHR-I primary antibody, raised against 18 C-terminal residues, diluted 1:200 in TBS containing 0.1% BSA, whereas the ovary sections were treated also with mouse monoclonal anti-GNRH primary antibody diluted as described above. The next day, the slides were rinsed three times in TBS for 5 min each, treated again with normal serum and then incubated for 30 min at room temperature with biotin rabbit anti-goat (for GNRHR-I) or biotin goat anti-rabbit (for GNRH-I) secondary antibodies diluted 1:200 in TBS. After TBS washes, the slides were exposed to ABC for 30 min and rinsed again with TBS. The peroxidase activity sites were visualized using DAB kit as chromogen; then the specimens were rinsed twice with distilled water for 5 min each, counterstained with hematoxylin, washed in running tap water and, finally, dehydrated passing through graded ethanol (70, 95, and 100% v/v), cleared in xylene and mounted with medium for light microscopy. Tissue sections in which the primary antibody was omitted or substituted by goat or mouse IgG were used as negative controls of non-specific staining. Pituitary tissue sections from a control rabbit were included as positive controls for the GNRHR-I reaction. The intensity of GNRHR-I and GNRH-I immunostaining in follicles and CL was assessed and compared microdensitometrically. The image analysis system (IAAS 2000 image analyzer, Delta Sistemi, Rome, Italy) was calibrated by taking the background developed in sections incubated with non-immune serum as 'zero' and a conventional value of maximum intensity of staining (Tomassoni *et al.* 2010) as '100'. Data are expressed in arbitrary units.

### GNRHR-I, FSHR, and LHR real-time RT-PCR

Total RNA was extracted from CL of three rabbits for each luteal stage as described previously (Boiti *et al.* 2005). According to the protocol provided by the manufacturer, 5 µg total RNA were reverse transcribed in 20 µl of iSCRIPT cDNA using random hexamer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. Serial experiments were performed to optimize the quantitative reaction, efficiency, and threshold cycle (*C<sub>t</sub>*) values. The optimal 25 µl PCR volume contained 12.5 µl iQ SYBR Green SuperMix, 1 µl forward and reverse primers (stock concentration 10 µmol/l), and 25 µl water. The primers used are listed in Table 1. All reagents were mixed as a master mix and distributed into a 96-well PCR plate before adding 2 µl cDNA (diluted 10-fold with water). For every PCR run were included reaction controls without template, as negative controls, and control without reverse transcriptase in RT, to assess whether the RNA was free by genomic DNA contamination. Samples' amplification fidelity was also verified by agarose gel electrophoresis. PCR was performed on iCycler iQ (Bio-Rad) with an initial incubation at 95 °C for 1.5 min, followed by 40 cycles at 95 °C for 15 s, 53 °C for 30 s, during which fluorescence data were collected. The *C<sub>t</sub>* value of each trace was automatically computed. PCR products were purified and sequenced by Qiaquick PCR Purification Kit according to the manufacturer's protocol. The 18S *C<sub>t</sub>* housekeeping gene, was determined to normalize sample variations in the amount of starting cDNA. Standard curves generated by plotting the *C<sub>t</sub>* value against the log cDNA standard dilution (1/10 dilution) in nuclease-free water were used to compare the relative amount of target genes. The slope of this graph was used to determine the reaction efficiency. Quantification of mRNA samples was performed by iCycler system software. The melting curve analysis, performed immediately after the PCR end cycle, was used to determine the specificity of each primer set. A melt-curve protocol was performed by repeating 80 °C heating for 10 s, from 55 °C with 0.5 °C increments, during which fluorescence data were collected. The melting temperatures of the products were determined graphically with fluorescence change rate (d(RFU)/dT) versus temperature.

**Table 1** Primers used for gene quantification by real-time PCR

Gene	Accession number		bp	Primers
<i>GNRHR-I</i>	AY781779 <i>Oryctolagus cuniculus</i>	Forward	191	TGATCCACCTCACAAATGGA
		Reverse		ATGAAGGACCCGTGTCAGAG
<i>FSHR</i>	AY429104.1 <i>Homo sapiens</i>	Forward	150	GAGGAATGCCATTGAACTGAGG
		Reverse		GGAAGGTTGGAGAACACATCTG
<i>LHR</i>	S57793 <i>Homo sapiens</i>	Forward	118	CTGGAGAAGATGCACAATGG
		Reverse		CAATTAGCCTCTGAATGGACTC
<i>18S</i>	X03205.1 <i>Homo sapiens</i>	Forward	148	CGATCAGATACCGTCGTAGT
		Reverse		TTCCTTTAAGTTTCAGCTTTGC

### Western blotting

Total luteal proteins were extracted from a pool of ten CL of three rabbits for each luteal stage. The CL were homogenized in 1 ml ice-cold RIPA buffer (PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS v/v) as described previously (Zerani *et al.* 2004). After incubation at 4 °C for 20 min, the homogenates were centrifuged at 12 000 *g* for 60 min at 4 °C. The supernatants were collected, and their protein concentrations were measured using the protein assay kit with BSA as a standard. Equal amounts of proteins (20 µg) were separated by discontinuous 10% SDS-PAGE (w/v) with 4% stacking gel (w/v) for 40 min at 200 V and 500 mA, after which proteins were transferred onto a nitrocellulose membrane for 1 h at 100 V and 350 mA. After the transfer, non-specific binding of antiserum was prevented by incubation with 0.05% Tween-20 (v/v), 5% non-fat dry milk (w/v), and 5% BSA (w/v) in TBS. Blocked membranes were then probed with antibody against GNRHR (1:500) overnight at 4 °C. After washing with TBS, the membranes were incubated with HRP-labeled rabbit anti-goat IgG secondary antibody (1:20 000) at room temperature for 1 h. All antibody incubations were performed in TBS containing 5% BSA (w/v) and 0.05% Tween-20 (v/v). After washing with TBS, the immunocomplexes were visualized by using an enhanced chemiluminescence detection kit according to the manufacturer's protocol and exposed to X-ray film. Blot images were acquired, and the intensities of the bands were quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories). Membranes were then reblotted with mouse anti-β-tubulin monoclonal antibody (0.05 µg/ml) and processed as described above to verify equal loading of proteins. Data were normalized by reference to β-tubulin after membrane stripping for 15 min with stripping solution. Values were expressed as arbitrary units of relative abundance of the specific protein normalized with that of β-tubulin used as a loading control.

### In vivo experiments

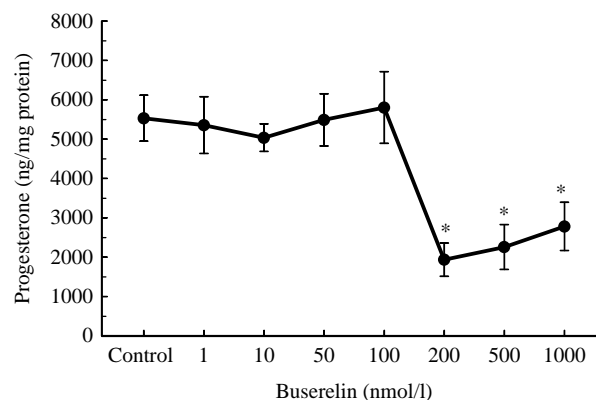
At days 4, 9, and 13 of pseudopregnancy, two groups of rabbits ( $n=6$ /group per day) were treated i.m. with either saline (0.2 ml) or buserelin (0.8 µg). From each rabbit, blood samples were collected by venipuncture of the marginal ear vein 0, 24, and 48 h after injection. The samples, collected in EDTA vacutainers, were centrifuged at 3000 *g* for 15 min, and the frozen plasma was stored until assayed for progesterone concentrations to assess the functional status of the ovarian CL. For the purposes of this work, functional luteolysis was defined as a 50% decrease in plasma progesterone from pre-treatment values, while complete luteolysis as the failure of CL to secrete progesterone so that blood levels fall below 1.0 ng/ml, which are found in estrous rabbits (Browning *et al.* 1980).

### In vitro experiments

A method reported previously was used for the *in vitro* study (Boiti *et al.* 2001). Day 4, 9, or 13 CL were randomly distributed (one CL/well) into incubation wells (Becton Dickinson Co., Clifton, NJ, USA) in 1 ml culture medium 199 with Earle's Balanced Salt Solution containing 2.2 mg/ml sodium bicarbonate, 2.3 mg/ml HEPES, and 3% BSA (w/v), referred to here as M199. Before treatment, the CL were quartered inside each well using fine forceps. Each set of incubation wells was divided into 12 experimental groups, each were formed of five wells as follows: I) medium alone as control; II) GNRH-I agonist (buserelin, 200 nmol/l); III) buserelin + GNRH-I antagonist (antide, 100 nmol/l); IV) buserelin + PLA2 inhibitor (4-bromophenacyl bromide, 2 µmol/l); V) buserelin + PLC inhibitor (compound 48/80, 2 µmol/l); VI) buserelin + PLD inhibitor (propranolol, 10 µmol/l); VII) buserelin + AC inhibitor (2-O-methyladenosine, 2 µmol/l); VIII) buserelin + inositol 1,4,5-trisphosphate (IP<sub>3</sub>) antagonist (2-aminoethyl diphenylborinate, 100 µmol/l); IX) buserelin + diacylglycerol (DAG) antagonist (1-hexadecyl-2-acetyl glycerol, 100 µmol/l); X) buserelin + protein kinase C (PKC) inhibitor (staurosporine 20 µmol/l); XI) buserelin + COX inhibitor (ASA, 100 µmol/l); XII) buserelin + NOS inhibitor (*N*-nitro-L-arginine methyl ester, 100 µmol/l). The culture plates were incubated at 37 °C in air with 5% CO<sub>2</sub>. The media of each well were collected after 4 h of incubation and stored immediately at -20 °C for later determination of progesterone, PGF<sub>2α</sub>, and PGE<sub>2</sub>. Preliminary evidence led us to choose the incubation conditions and the minimum effective dose for buserelin used in the present *in vitro* study (Fig. 1).

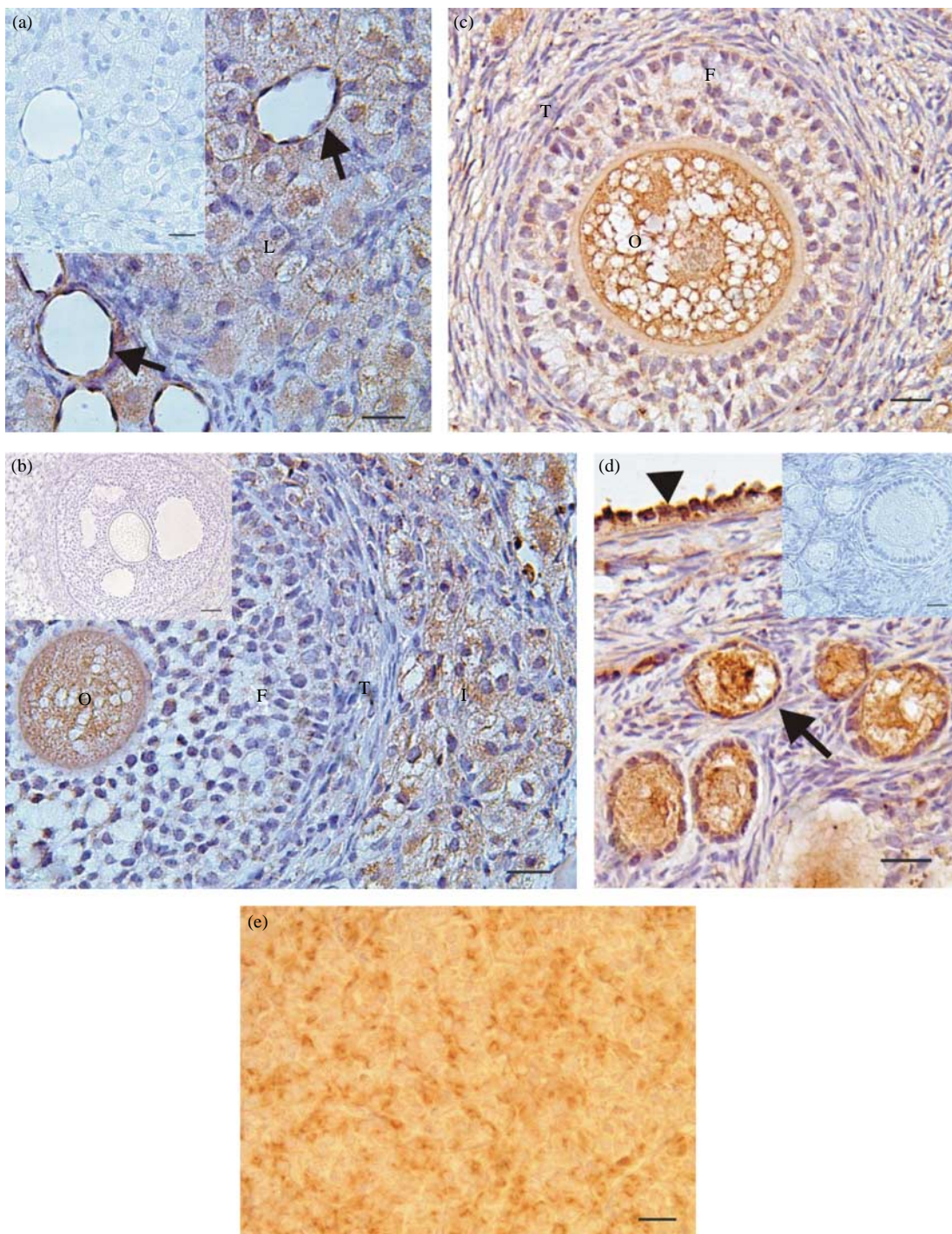
### Hormone determination

Progesterone, PGF<sub>2α</sub>, and PGE<sub>2</sub> were determined following the RIA protocols reported previously (Boiti *et al.* 2001). Intra- and inter-assay coefficients of variation and minimum



**Figure 1** *In vitro* effects of increasing concentrations of GNRH-I agonist (buserelin) on progesterone release by rabbit CL collected at day 9 of pseudopregnancy. Values are means  $\pm$  s.d. of five replicates. Asterisks indicate a significantly different value ( $P < 0.01$ ) versus control.





**Figure 2** Immunostaining for GNRHR-I of rabbit ovaries during the mid stage of pseudopregnancy. (a) The immunopositivity is evident in the cytoplasm of luteal cells (L) and endothelial cells (arrows), (insert: control section in the absence of primary antibody); (b) immunosignals are detected in the ooplasm of oocytes (O) and cytoplasm of follicular (F), thecal cells (T), and endocrine interstitial cells (I) present in antral follicles, (insert: control section); (c) the immunolabeling is localized in the ooplasm of oocytes (O) and cytoplasm of follicular (F) and thecal cells (T) present in pre-antral follicles; (d) immunosignals are evident in the ooplasm of oocytes and cytoplasm of follicular cells present in primordial follicles (arrow) and in ovarian epithelium (arrowhead), (insert: control section); (e) rabbit pituitary gland: cell groups show an intense immunoreactivity for GNRHR-I. Scale bar = 20 μm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0109>.

detectable dose were progesterone: 5 and 9%, 10 pg; PGF<sub>2α</sub>: 8 and 12%, 19 pg; PGE<sub>2</sub>: 7 and 13%, 18 pg.

#### Enzyme activity determination

Luteal COX-1 and -2 activities were determined by measuring the disappearance of the radiolabeled substrate [<sup>3</sup>H]AA using a method reported previously (Zerani *et al.* 2007). Selective COX-2 inhibitor (NS-398 1 μmol/l) and non-selective COX inhibitor (ASA, 1 mmol/l) were used. For each sample, COX-1 activity was determined by calculating the rate of loss of [<sup>3</sup>H]AA incubated with selective COX-2 inhibitor. Conversely, the COX-2 activity of each corresponding sample was determined by calculating the rate of loss of [<sup>3</sup>H]AA incubated without selective COX-2 inhibitor and subtracting from this value that of COX-1. The values for COX-1 and -2 were corrected by subtracting the [<sup>3</sup>H]AA disappearance values due to other enzymatic activities and to non-enzymatic reactions.

NOS activity was determined by monitoring the conversion of [<sup>3</sup>H]L-arginine to [<sup>3</sup>H]L-citrulline with a commercial NOS assay kit, according to the experimental protocol described by Boiti *et al.* (2000).

#### Statistical analysis

Data on gene and protein expressions, hormone levels, and enzyme activities were examined by ANOVA followed by the Student–Newman–Keuls *t*-test. Differences were considered significant at  $P < 0.01$ .

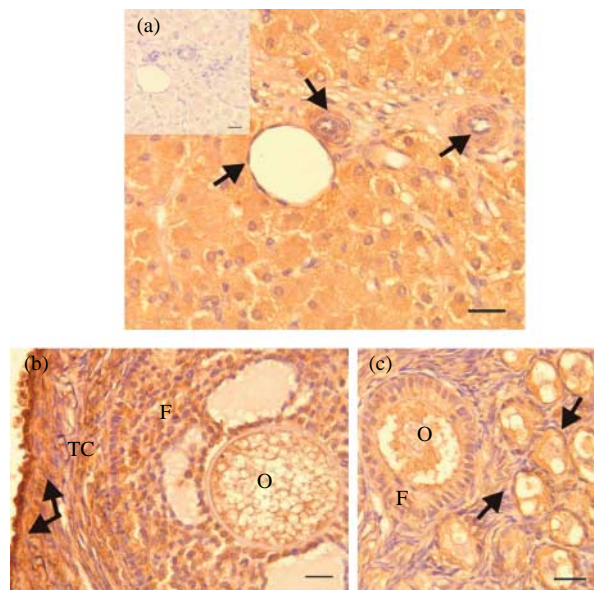
## Results

#### *GNRHR-I and GNRH-I immunolocalization*

**GNRHR-I** Immunopositivity for GNRHR-I was detected in the cytoplasm of luteal cells and endothelial cells (Fig. 2, photo a); additionally, it was detected in the ooplasm of oocytes and cytoplasm of follicular and thecal cells present both in antral and pre-antral follicles (Fig. 2, photos b and c). Immunosignals were also evident in the ooplasm of oocytes and cytoplasm of follicular cells present in primordial follicles (Fig. 2, photo d), ovarian epithelium (Fig. 2, photo d), and endocrine interstitial cells (Fig. 2, photo a). The intervening fibroblast cells within the CL were always unreactive. Differences in the presence and intensity of immunostaining were not observed during the three luteal stages. The control staining procedure failed to disclose appreciable reactivity at any of the sites described for GNRHR-I (inserts of Fig. 2, photos a, b and d). The cytoplasm of pituitary cells exhibited intense immunostaining (Fig. 2, photo e). In the ovary collected at day 4 of pseudopregnancy, the intensity of GNRHR-I immunostaining was higher in CL than in follicles ( $39.92 \pm 5.46$  and  $29.53 \pm 3.99$  respectively,

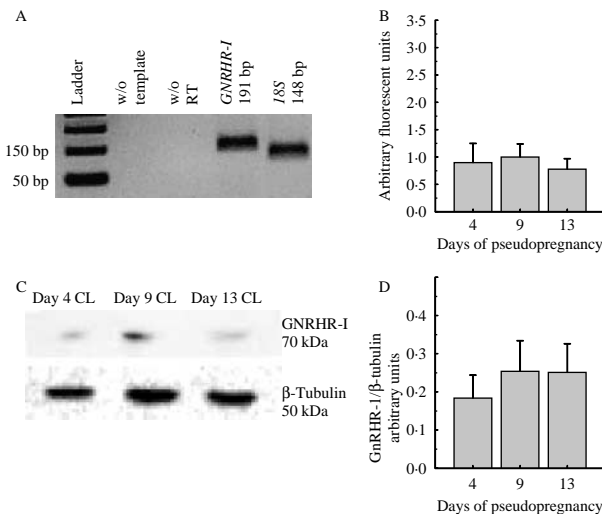
$P < 0.01$ ), whereas no differences were found in the ovaries at later stages of pseudopregnancy (data not shown).

**GNRH-I** Positive immunosignals for GNRH-I were evidenced in the cytoplasm of luteal cells at all stages of pseudopregnancy (Fig. 3, photo a). The cytoplasm of follicular and thecal cells and the ooplasm within antral follicles were immunopositive (Fig. 3, photo b) as well as the follicular cells and the ooplasm within pre-antral and primordial follicles (Fig. 3, photo c). The ovarian epithelium (Fig. 3, photo b), endothelial cells (Fig. 3, photo a), and interstitial cells displayed an intense immunoreactivity. The connective tissue within the CL was not immunostained, whereas that of the ovarian cortex immunoreacted to GNRH-I (Fig. 3, photo c). The control sections were not stained at any sites examined for GNRH-I (insert of Fig. 3, photo b). The intensity of GNRH-I immunostaining did not show any difference between CL and follicles at any luteal stage considered (data not shown).



**Figure 3** Immunostaining for GNRH-I of rabbit ovaries during the mid stage of pseudopregnancy. (a) Immunoreactivity is intensely evidenced in the cytoplasm of luteal cells; the endothelial cells (arrows) are immunolabeled; (b) the cytoplasm of follicular (F) and thecal cells (TC) and the ooplasm (O) within antral follicles are intensely immunopositive; the ovarian epithelium is strongly immunostained; insert, control section in which the primary antibody was omitted does not evidence immunopositivity at any of the sites described for GNRH-I; (c) the follicular cells (F) and the ooplasm (O) within pre-antral and primordial follicles are intensely immunostained; note the immunopositivity of the connective tissue present in the ovarian cortical cells (arrows). Scale bar = 20 μm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0109>.





**Figure 4** (A) Gene expression of *GNRHR-I* mRNA in CL of rabbits collected at days 4, 9, and 13 of pseudopregnancy. Representative agarose gel electrophoresis stained with ethidium bromide to verify matching between expected and obtained PCR products. For every PCR, two negative controls were included, without template and without reverse transcriptase respectively. (B) The values, expressed in arbitrary fluorescence units related to *18S*, are means  $\pm$  s.d. of four independent replicates. (C) Immunoblot showing GNRHR-I in luteal CL lysates collected at days 4, 9, and 13 of pseudopregnancy. Total protein extracts were separated by SDS gel electrophoresis and analyzed by immunoblotting with GNRHR-I-specific antibody. (D) Densitometric analysis of bands corresponding to GNRHR-I after WB. Values of GNRHR-I protein bands were standardized to those of  $\beta$ -tubulin. The results are means  $\pm$  s.d. of four independent experiments.

#### Expression of mRNA and protein for luteal GNRHR-I, FSHR, and LHR

*GNRHR-I* transcript was expressed in CL at different stages of pseudopregnancy with no changes in its relative abundance (Fig. 4, panels A and B). The protein abundance of GNRHR-I did not differ between days 4, 9, and 13 of pseudopregnancy (Fig. 4, panels C and D). The expressions of *FSHR* and *LHR* transcripts were higher ( $P < 0.01$ ) in day 13 CL than in day 4 and 9 CL (Fig. 5).

#### In vivo experiments

Buserelin administration at days 9 and 13 of pseudopregnancy decreased ( $P < 0.01$ ) the peripheral plasma progesterone concentrations for 48 and 24 h respectively (Fig. 6), whereas buserelin injection at day 4 did not affect progesterone levels (Fig. 6, panel B).

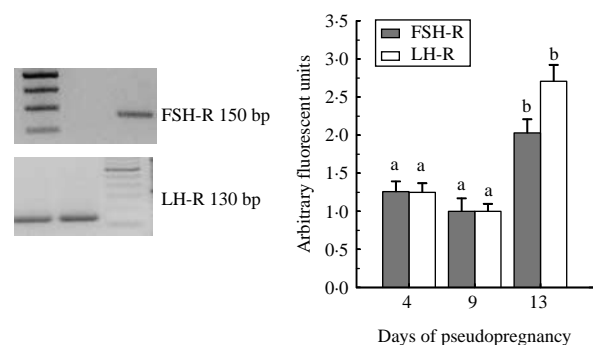
#### In vitro experiments

**Hormone releases** Basal *in vitro* progesterone secretion was higher in day 9 CL ( $P < 0.01$ ) than in day 4 and 13 CL, secretion in day 13 CL was higher ( $P < 0.01$ ) than in day 4 CL

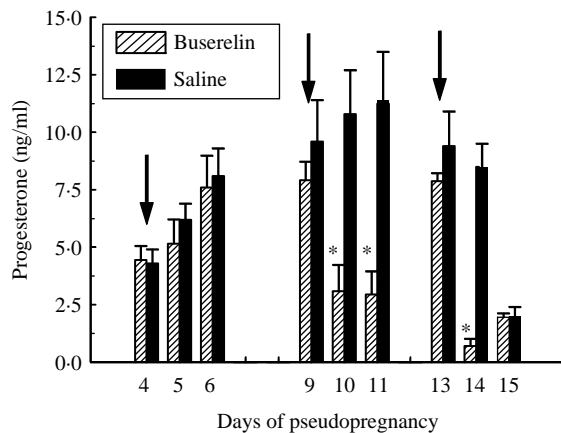
(Fig. 7, top panel). Buserelin reduced ( $P < 0.01$ ) progesterone *in vitro* secretion by both day 9 and 13 CL, but had no effect on day 4 CL (Fig. 7, top panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted ( $P < 0.01$ ) the effects of buserelin alone on day 9 and 13 CL (Fig. 7, top panel). The co-treatments with ASA or *N*-nitro-L-arginine methyl ester inhibitor increased ( $P < 0.01$ ) progesterone secretion by both day 9 and 13 CL (Fig. 7, top panel). The co-treatment with 4-bromophenacyl bromide, propranolol, or 2-*O*-methyladenosine did not affect the buserelin-decreased progesterone secretion on day 9 and 13 CL (data not shown).

Basal *in vitro*  $\text{PGF}_{2\alpha}$  secretion was higher in day 13 CL ( $P < 0.01$ ) than in day 4 and 9 CL, and secretion in day 9 CL was higher ( $P < 0.01$ ) than in day 4 CL (Fig. 7, bottom panel). Buserelin increased ( $P < 0.01$ )  $\text{PGF}_{2\alpha}$  *in vitro* secretion by both day 9 and 13 CL (Fig. 7, bottom panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted ( $P < 0.01$ ) the effects of buserelin alone on day 9 and 13 CL (Fig. 7, bottom panel). The co-treatment with ASA decreased ( $P < 0.01$ )  $\text{PGF}_{2\alpha}$  secretion in all CL types, while the co-treatment with *N*-nitro-L-arginine methyl ester did not affect the buserelin-induced  $\text{PGF}_{2\alpha}$  secretion (Fig. 7, bottom panel).

Basal *in vitro*  $\text{PGE}_2$  secretion was higher in day 4 CL ( $P < 0.01$ ) than in day 9 and 13 CL (Fig. 7, middle panel). Buserelin reduced ( $P < 0.01$ )  $\text{PGE}_2$  *in vitro* secretion by day 13 CL (Fig. 7, middle panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted ( $P < 0.01$ ) the effects of buserelin alone on day 13 CL (Fig. 7, middle panel). The co-treatment with ASA decreased ( $P < 0.01$ )  $\text{PGE}_2$  secretion in



**Figure 5** (A) Gene expressions of *FSH* and *LH* mRNA in CL of rabbits collected at days 4, 9, and 13 of pseudopregnancy. Representative agarose gel electrophoresis stained with ethidium bromide to verify matching between expected and obtained PCR products. For every PCR, two negative controls were included, without template and without reverse transcriptase respectively. (B) The values, expressed in arbitrary fluorescence units related to *18S*, are means  $\pm$  s.d. of four independent replicates. Different letters above the bars indicate significantly different values ( $P < 0.01$ ).



**Figure 6** Plasma progesterone levels in pseudopregnant rabbits 0, 24, and 48 h after the injection with saline or GNRH analog (buserelin) at days 4, 9, or 13 of pseudopregnancy. The black arrows point out the injection day. Values are mean  $\pm$  s.d. of six replicates. Asterisks indicate significantly different values versus saline ( $P < 0.01$ ).

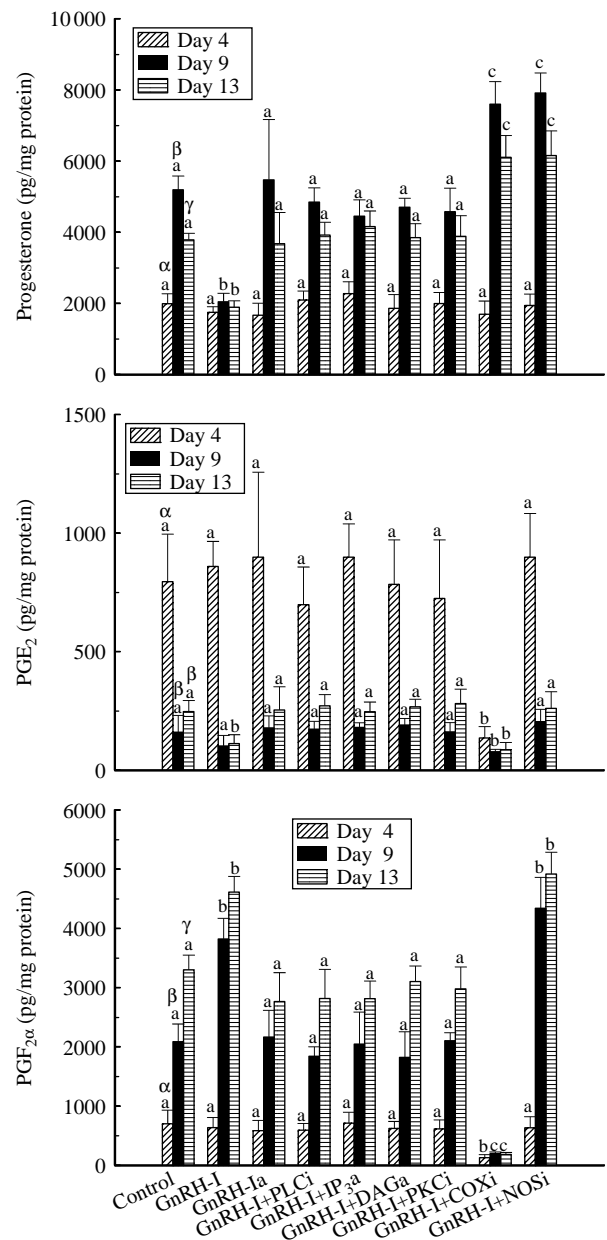
all CL types, while the co-treatment with *N*-nitro-*L*-arginine methyl ester did not affect the buserelin-induced  $\text{PGE}_2$  secretion (Fig. 7, middle panel).

**Enzyme activities** Basal COX-1 activity was higher in day 13 CL ( $P < 0.01$ ) than in day 4 and 9 CL (Fig. 8, top panel). All treatments did not affect COX-1 activity in all CL types, except for ASA that decreased ( $P < 0.01$ ) the enzymatic activity (Fig. 8, top panel).

Basal COX-2 activity of day 13 CL was higher ( $P < 0.01$ ) than that of day 4 and 9 CL; COX-2 activity of day 9 CL was higher ( $P < 0.01$ ) than that of day 4 CL (Fig. 8, middle panel). Buserelin increased ( $P < 0.01$ ) COX-2 activity in CL of all stages (Fig. 8, middle panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted ( $P < 0.01$ ) the effects of buserelin alone (Fig. 8, middle panel). The co-treatments with ASA decreased ( $P < 0.01$ ) COX-2 activity in all CL types, while the co-treatments with *N*-nitro-*L*-arginine methyl ester did not affect the buserelin-induced COX-2 activity increase (Fig. 8, middle panel).

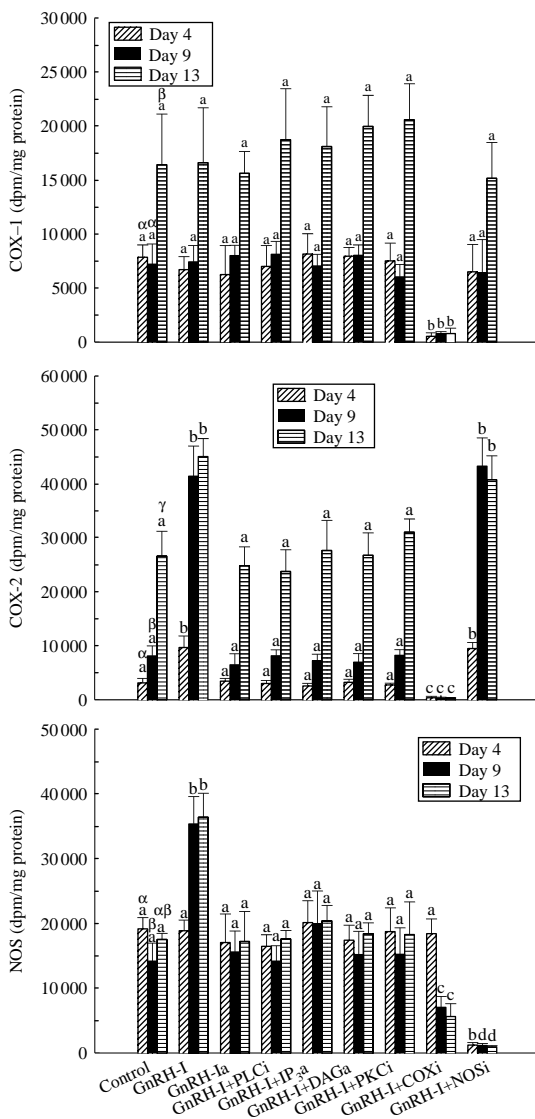
Basal NOS activity of day 4 CL was higher ( $P < 0.01$ ) than that of day 9 CL (Fig. 8, bottom panel). Buserelin increased ( $P < 0.01$ ) NOS activity in both day 9 and 13 CL (Fig. 8, bottom panel). The co-incubation of buserelin with antide, compound 48/80, 2-aminoethyl diphenylborinate, 1-hexadecyl-2-acetyl glycerol, or staurosporine counteracted ( $P < 0.01$ ) the effects of buserelin alone at day 9 and 13 (Fig. 8, bottom panel). The co-treatments with *N*-nitro-*L*-arginine methyl ester decreased ( $P < 0.01$ ) NOS activity in CL of all stages, while that with ASA in both day 9 and 13 CL (Fig. 8, bottom panel).

All hormone release and enzyme activity data are summarized in the synoptic Table 2.



**Figure 7** *In vitro* effects of GNRH-I agonist (buserelin, GNRH-I) alone or co-incubated with antagonists for GNRH-I (antide, GNRH-Ia),  $\text{IP}_3$  (2-aminoethyl diphenylborinate,  $\text{IP}_3\text{a}$ ), and DAG (1-hexadecyl-2-acetyl glycerol, DAGa), or inhibitors for PLC (compound 48/80, PLCi), PKC (staurosporine, PKCi), COX (acetylsalicylic acid, COXi), and NOS (*N*-nitro-*L*-arginine methyl ester, NOSi) on progesterone (top panel),  $\text{PGE}_2$  (middle panel), and  $\text{PGF}_{2\alpha}$  (bottom panel) release by rabbit CL at days 4, 9, and 13 of pseudopregnancy. Values are means  $\pm$  s.d. of five replicates. Different letters above the bars indicate significantly different values ( $P < 0.01$ ): Greek letters among control CL days; Latin letters among experimental groups of the same CL day.





**Figure 8** *In vitro* effects of GNRH-I agonist (buserelin, GNRH-I) alone or co-incubated with antagonists for GNRH-I (antide, GNRH-Ia), IP<sub>3</sub> (2-aminoethyl diphenylborinate, IP<sub>3</sub>a), and DAG (1--hexadecyl-2-acetyl glycerol, DAGa), or inhibitors for PLC (compound 48/80, PLCi), PKC (staurosporine, PKCi), COX (acetylsalicylic acid, COXi), and NOS (N-nitro-L-arginine methyl ester, NOSi) on COX-1 (top panel), COX-2 (middle panel), and NOS (bottom panel) activities by rabbit CL at days 4, 9, and 13 of pseudopregnancy. Values are means  $\pm$  s.d. of five replicates. Different letters above the bars indicate significantly different values ( $P < 0.01$ ): Greek letters among control CL days; Latin letters among experimental groups of the same CL day.

## Discussion

The present study, for the first time, indicates the presence of a GNRH/GNRHR system that modulates the steroidogenic luteal function of pseudopregnant rabbits.

The first evidence for the presence of ovarian GNRHR, through ligand-specific binding sites, was observed in rat granulosa and luteal cells (Reeves *et al.* 1980, Pieper *et al.* 1981, Olofsson *et al.* 1995). Subsequently, the presence of GNRHR mRNA was also identified in human granulosa luteal cells (Kang *et al.* 2000) and in both follicles and CL of bovine ovary (Ramakrishnappa *et al.* 2001). Among the different stages of CL, GNRHR transcripts were clearly detectable in stage III luteal tissues, whereas the expression levels were slightly lower in stage II, and barely or no detectable signal was evident from stages I and IV luteal tissues (Milvae *et al.* 1984).

Our study showed the presence of GNRHR-I at both transcript and protein levels in CL of pseudopregnant rabbits. Apparently, both mRNA and protein abundances did not change at different luteal stages, suggesting that in the rabbit this receptor is not dynamically regulated during the CL lifespan. In both early- and mid-luteal stages, luteal GNRHR-I, FSHR, and LHR transcript levels were expressed at approximately the same relative abundance. By converse, the GNRHR-I to FSHR or to LHR mRNAs ratio decreased in CL of late-luteal stage as a consequence of the two- to three-fold up-regulation of these two ovarian markers. Positive staining for GNRHR-I was clearly evidenced in the cytoplasm of luteal cells during all stages of pseudopregnancy.

The presence of GNRH-I has been revealed in the ovary of rats (Aten *et al.* 1986, Schirman-Hildelsheim *et al.* 2005, Sengupta *et al.* 2008), monkeys (Chakrabarti *et al.* 2008), and humans (Aten *et al.* 1987). The present investigation confirms that also the ovary of rabbits expresses GNRH-I in the cytoplasm of different cell types, including the luteal cells, independently of the luteal stage examined.

The presence of GNRHR-I and GNRH-I, similarly to what found in other species, supports a direct role of GNRH in the regulation of luteal functions in rabbits. GNRHR-I immunoreactivity was also observed in other rabbit ovary cells as oocytes, follicular and thecal cells, so stressing the potential paracrine and/or autocrine role of the GNRH/GNRHR system in modulating ovary function in mammals (Ramakrishnappa *et al.* 2005). Interestingly, in the early-luteal stage, the microdensitometry results revealed the presence of larger GNRHR-I amounts in luteal than in follicle cells suggesting that GNRH-I is involved also in the luteotropic processes.

GNRH has been shown to exert a mixture of both inhibitory and stimulatory effects in the gonads, causing either inhibitory or stimulatory effects on ovarian cellular steroid output (Ramakrishnappa *et al.* 2005). In bovine, two GNRH antagonists, buserelin and deslorelin, showed *in vivo* and *in vitro* inhibitory effects on progesterone release (Milvae *et al.* 1984, D'Occhio & Aspden 1999). Conversely, GNRH stimulatory *in vitro* effects on progesterone production were demonstrated in monkey and human granulosa cells (Casper *et al.* 1984, Liu *et al.* 1991, Bussenot *et al.* 1993). Finally, other authors documented that GNRH has no effect on progesterone production in human granulosa-lutein cells (Casper *et al.* 1984). In rabbits, GNRH agonist directly affected the

**Table 2** *In vitro* effects of GNRH-I agonist (buserelin, GNRH-I) alone or co-incubated with antagonists for GNRH-I (antide, GNRH-Ia), IP<sub>3</sub> (2-aminoethyl diphenylborinate, IP<sub>3</sub>a), and DAG (1-hexadecyl-2-acetyl glycerol, DAGa), or inhibitors for PLC (compound 48/80, PLCi), PKC (staurosporine, PKCi), COX (acetylsalicylic acid, COXi), and NOS (N-nitro-L-arginine methyl ester, NOSi) on progesterone, PGE<sub>2</sub>, and PGF<sub>2α</sub> release and COX-1, COX-2, and NOS activities by rabbit CL at days 4, 9, and 13 of pseudopregnancy. Values are means ± s.d. of five replicates

	Progesterone	PGF <sub>2α</sub>	PGE <sub>2</sub>	COX-1	COX-2	NOS
<b>Day 4</b>						
Control	1997 ± 273 <sup>αα</sup>	707 ± 225 <sup>αα</sup>	796 ± 200 <sup>αα</sup>	7859 ± 1135 <sup>αα</sup>	3145 ± 825 <sup>αα</sup>	19 202 ± 1702 <sup>αα</sup>
GNRH-I	1755 ± 152 <sup>a</sup>	640 ± 167 <sup>a</sup>	860 ± 105 <sup>a</sup>	6728 ± 1178 <sup>a</sup>	9705 ± 2101 <sup>b</sup>	18 910 ± 1613 <sup>a</sup>
GNRH-I + GNRH-Ia	1671 ± 336 <sup>a</sup>	702 ± 172 <sup>a</sup>	899 ± 358 <sup>a</sup>	6264 ± 2667 <sup>a</sup>	3478 ± 492 <sup>a</sup>	17 096 ± 4393 <sup>a</sup>
GNRH-I + PLCi	2099 ± 248 <sup>a</sup>	597 ± 112 <sup>a</sup>	699 ± 158 <sup>a</sup>	7030 ± 1912 <sup>a</sup>	3044 ± 508 <sup>a</sup>	16 475 ± 1788 <sup>a</sup>
GNRH-I + IP <sub>3</sub> a	2280 ± 332 <sup>a</sup>	715 ± 183 <sup>a</sup>	899 ± 140 <sup>a</sup>	8152 ± 1881 <sup>a</sup>	2587 ± 437 <sup>a</sup>	20 172 ± 3336 <sup>a</sup>
GNRH-I + DAGa	1860 ± 389 <sup>a</sup>	627 ± 114 <sup>a</sup>	785 ± 187 <sup>a</sup>	7961 ± 803 <sup>a</sup>	3219 ± 567 <sup>a</sup>	17 438 ± 2315 <sup>a</sup>
GNRH-I + PKCi	2001 ± 305 <sup>a</sup>	617 ± 152 <sup>a</sup>	725 ± 247 <sup>a</sup>	7526 ± 1640 <sup>a</sup>	2746 ± 305 <sup>a</sup>	18 762 ± 3655 <sup>a</sup>
GNRH-I + COXi	1696 ± 371 <sup>a</sup>	133 ± 47 <sup>b</sup>	137 ± 47 <sup>b</sup>	537 ± 323 <sup>b</sup>	372 ± 252 <sup>c</sup>	18 472 ± 2255 <sup>a</sup>
GNRH-I + NOSi	1947 ± 317 <sup>a</sup>	637 ± 185 <sup>a</sup>	899 ± 184 <sup>a</sup>	6509 ± 2542 <sup>a</sup>	9497 ± 1072 <sup>b</sup>	1246 ± 340 <sup>b</sup>
<b>Day 9</b>						
Control	5197 ± 383 <sup>αβ</sup>	2089 ± 297 <sup>αβ</sup>	161 ± 71 <sup>αβ</sup>	7220 ± 1855 <sup>αα</sup>	8116 ± 1830 <sup>αβ</sup>	14 212 ± 2764 <sup>αβ</sup>
GNRH-I	2048 ± 240 <sup>b</sup>	3825 ± 346 <sup>b</sup>	103 ± 44 <sup>a</sup>	7442 ± 1505 <sup>a</sup>	41 452 ± 5559 <sup>b</sup>	35 421 ± 4201 <sup>b</sup>
GNRH-I + GNRH-Ia	5479 ± 1693 <sup>a</sup>	2170 ± 447 <sup>a</sup>	179 ± 50 <sup>a</sup>	8013 ± 962 <sup>a</sup>	6472 ± 2044 <sup>a</sup>	15 608 ± 3236 <sup>b</sup>
GNRH-I + PLCi	4851 ± 394 <sup>a</sup>	1842 ± 160 <sup>a</sup>	174 ± 32 <sup>a</sup>	8135 ± 1197 <sup>a</sup>	8135 ± 1094 <sup>a</sup>	14 197 ± 2369 <sup>b</sup>
GNRH-I + IP <sub>3</sub> a	4458 ± 452 <sup>a</sup>	2051 ± 536 <sup>a</sup>	181 ± 19 <sup>a</sup>	7061 ± 1073 <sup>a</sup>	7213 ± 1241 <sup>a</sup>	19 971 ± 5048 <sup>b</sup>
GNRH-I + DAGa	4708 ± 250 <sup>a</sup>	1825 ± 431 <sup>a</sup>	191 ± 27 <sup>a</sup>	8048 ± 951 <sup>a</sup>	6956 ± 1586 <sup>a</sup>	15 230 ± 3553 <sup>b</sup>
GNRH-I + PKCi	4580 ± 664 <sup>a</sup>	2107 ± 133 <sup>a</sup>	162 ± 39 <sup>a</sup>	6053 ± 1139 <sup>a</sup>	8237 ± 1039 <sup>a</sup>	15 246 ± 4083 <sup>b</sup>
GNRH-I + COXi	7604 ± 633 <sup>c</sup>	202 ± 27 <sup>c</sup>	78 ± 10 <sup>b</sup>	767 ± 197 <sup>b</sup>	318 ± 144 <sup>c</sup>	7065 ± 1667 <sup>c</sup>
GNRH-I + NOSi	7919 ± 558 <sup>c</sup>	4343 ± 521 <sup>b</sup>	205 ± 52 <sup>a</sup>	6436 ± 3051 <sup>a</sup>	43 295 ± 5218 <sup>b</sup>	1158 ± 304 <sup>d</sup>
<b>Day 13</b>						
Control	3789 ± 182 <sup>αγ</sup>	3304 ± 245 <sup>αγ</sup>	248 ± 46 <sup>αβ</sup>	16 440 ± 5308 <sup>αβ</sup>	26 599 ± 4624 <sup>αγ</sup>	17 549 ± 950 <sup>ααβ</sup>
GNRH-I	1894 ± 176 <sup>b</sup>	4614 ± 266 <sup>b</sup>	113 ± 37 <sup>b</sup>	16 636 ± 5068 <sup>a</sup>	45 060 ± 3325 <sup>b</sup>	36 432 ± 3697 <sup>b</sup>
GNRH-I + GNRH-Ia	3680 ± 877 <sup>a</sup>	2768 ± 485 <sup>a</sup>	255 ± 97 <sup>a</sup>	15 640 ± 2021 <sup>a</sup>	24 809 ± 3494 <sup>a</sup>	17 212 ± 4669 <sup>a</sup>
GNRH-I + PLCi	3922 ± 356 <sup>a</sup>	2819 ± 492 <sup>a</sup>	272 ± 47 <sup>a</sup>	18 757 ± 4722 <sup>a</sup>	23 776 ± 4026 <sup>a</sup>	17 616 ± 1326 <sup>a</sup>
GNRH-I + IP <sub>3</sub> a	4165 ± 434 <sup>a</sup>	2816 ± 296 <sup>a</sup>	247 ± 41 <sup>a</sup>	18 126 ± 3678 <sup>a</sup>	27 674 ± 5558 <sup>a</sup>	20 435 ± 2380 <sup>a</sup>
GNRH-I + DAGa	3850 ± 391 <sup>a</sup>	3104 ± 262 <sup>a</sup>	268 ± 31 <sup>a</sup>	19 989 ± 2879 <sup>a</sup>	26 756 ± 4166 <sup>a</sup>	18 426 ± 1684 <sup>a</sup>
GNRH-I + PKCi	3892 ± 576 <sup>a</sup>	2981 ± 369 <sup>a</sup>	282 ± 60 <sup>a</sup>	20 608 ± 3325 <sup>a</sup>	31 061 ± 2442 <sup>a</sup>	18 326 ± 5029 <sup>a</sup>
GNRH-I + COXi	6108 ± 613 <sup>c</sup>	185 ± 34 <sup>c</sup>	86 ± 31 <sup>b</sup>	796 ± 482 <sup>b</sup>	292 ± 71 <sup>c</sup>	5640 ± 1982 <sup>c</sup>
GNRH-I + NOSi	6161 ± 693 <sup>c</sup>	4922 ± 366 <sup>b</sup>	261 ± 70 <sup>a</sup>	15 182 ± 3319 <sup>a</sup>	40 792 ± 4471 <sup>b</sup>	963 ± 164 <sup>d</sup>

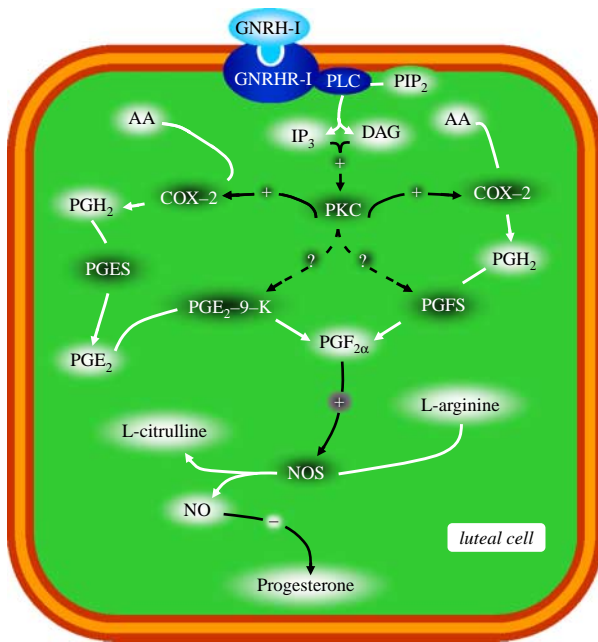
Different letters indicate significantly different values ( $P < 0.01$ ): Greek letters among control CL days; Latin letters among experimental groups of the same CL day.

ovary by inducing ovulation in both hypophysectomized animals and *in vitro* perfused system (Koos & LeMaire 1985). At day 11 of pseudopregnancy, GNRH injection induced luteinization of ovarian follicles and functional regression of CL as assessed by decline in serum progesterone levels within 3 days (Rippel *et al.* 1976). Depending on the dosage administered between days 8 and 13 of gestation, GNRH was found to either cause abortion by luteolysis or support fetal survival by stimulating luteotropic activity (Eisenberg *et al.* 1984). Conversely, other authors found that GNRH did not exhibit any direct inhibitory effect on steroid production in extracorporeal perfused ovary (Eisenberg *et al.* 1984).

Our data strengthen the idea that GNRH-I has a direct role in the down-regulation of rabbit CL progesterone production. The *in vitro* experiments showed that buserelin reduced progesterone production by cultured CL collected at days 9 and 13 of pseudopregnancy, whereas this steroid synthesis was not affected by GNRH-I agonist in day 4 CL. In addition, this GNRH-I role is also supported by the data of the co-incubation with antide, a GNRH-I antagonist, that counteracted the buserelin-induced progesterone decrease.

The *in vivo* experiments mirrored the above-reported *in vitro* data; in fact, blood progesterone levels were down-regulated when buserelin was administered at mid- and late-pseudopregnancy. Even if we cannot exclude that this *in vivo* buserelin-induced circulating progesterone decrease is also caused by a pituitary gonadotropin down-regulation, the *in vivo* and *in vitro* coincident lack of buserelin effects during early-pseudopregnancy suggests that this progesterone decline is primarily or exclusively due to the direct action of GNRH-I agonist on pseudopregnant CL.

Recently, we demonstrated, through *in vitro* studies on CL of pseudopregnant rabbits, that PGF<sub>2α</sub> and PGE<sub>2</sub> affect progesterone release differently, depending on the luteal stage (Boiti *et al.* 2001). PGE<sub>2</sub> depressed NOS activity and increased progesterone production in day 4 CL but was totally ineffective in day 9 CL; on the contrary, PGF<sub>2α</sub> up-regulated NOS activity and induced functional luteolysis in day 9 CL but had no effect on CL collected at the early-luteal phase. This physiological mechanism protects the growing CL from functional luteolysis to occur in the early-luteal stage until day 6 of pseudopregnancy, when CL shift from refractoriness



**Figure 9** Schematic representation of the suggested GNRH-I post-receptor mechanism regulating the progesterone production in rabbit CL. The hatched lines represent other possible PKC target(s). AA, arachidonic acid; COX-2, cyclo-oxygenase-2; DAG, diacylglycerol; GNRHR-I, type I GNRH receptor; IP<sub>3</sub>, inositol trisphosphate; NO, nitric oxide; NOS, nitric oxide synthase; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PGE<sub>2</sub>-9-K, prostaglandin E<sub>2</sub>-9-ketoreductase; PGF<sub>2α</sub>, prostaglandin F<sub>2α</sub>; PGFS, prostaglandin F<sub>2α</sub> synthase; PGH<sub>2</sub>, prostaglandin H<sub>2</sub>; PIP<sub>2</sub>, phosphatidylinositol diphosphate; PKC, protein kinase C; PLC, phospholipase C. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0109>.

to partial responsiveness to exogenous PGF<sub>2α</sub>, and acquire luteolytic competence (Boiti *et al.* 1998, 2001, 2003). The data on the buserelin ineffectiveness at early-luteal stage suggest that this luteolytic-protecting mechanism includes also the GNRH-I/GNRHR-I system, at least *in vitro*.

Many studies indicate that GNRH, complexing with cognate receptor, mainly activates PLC via G<sub>q/11</sub> family G proteins. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP<sub>3</sub> and DAG (Millar 2005). Our data confirmed that this GNRH post-receptor mechanism is present also in the rabbit CL. In fact, the effects of buserelin in decreasing progesterone release on day 9 and 13 CL were counteracted by the co-incubation with PLC and PKC inhibitors or IP<sub>3</sub> and DAG antagonists, while the inhibitors of AC, PLA<sub>2</sub>, and PLD had no influences.

A recent study showed that in the gonadotrope cell line LβT2, GNRH produced a marked time-dependent induction of COX-2 expression and an increase in PGE<sub>2</sub> and PGF<sub>2α</sub> production (Naor *et al.* 2007). These authors hypothesize a novel GNRH signaling pathway mediated by PGF<sub>2α</sub>, which acts through an autocrine/paracrine modality. The present work suggests that the GNRH-I effects on rabbit

CL progesterone production are mediated by the activation of the COX-2/PGF<sub>2α</sub>/NOS pathway. In fact, buserelin increased *in vitro* COX-2 activity, PGF<sub>2α</sub> production, and NOS activity in both day 9 and 13 CL, coinciding with decreasing progesterone release. In addition, the co-treatment with antide, PLC and PKC inhibitors, or IP<sub>3</sub> and DAG antagonists counteracted the buserelin actions on COX-2, PGF<sub>2α</sub>, NOS, and progesterone, while COX inhibitor counteracted those on NOS and progesterone, and, in turn, NOS inhibitor counteracted those on progesterone only. The decrease in PGE<sub>2</sub> induced by buserelin in day 13 CL is probably due to the increased conversion of this PG into PGF<sub>2α</sub> through PGE<sub>2</sub>-9-ketoreductase enzyme activation and/or the inactivation of PGE<sub>2</sub> synthase. A similar intracellular mechanism was reported for the PGF<sub>2α</sub> auto-amplifying loop utilized by the rabbit CL during the luteolytic process (Zerani *et al.* 2007). Our results also indicated that early CL refractoriness to luteolytic factors extends to GNRH-I as well, given that the all *in vitro* effects of buserelin on hormone productions and enzymatic activities occur only in day 9 and 13 CL.

In conclusion, this study provides the evidence that GNRH-I, in autocrine, paracrine, and/or endocrine manner, directly down-regulates progesterone production in rabbit CL that have acquired luteolytic competence, with a receptorial/post-receptorial mechanism as summarized in Fig. 9. GNRH-I couples its cognate type I receptor activating PLC and, in turn, releasing IP<sub>3</sub> and DAG; these two intracellular messengers, through the PKC activation, start up COX-2 activity, with the consequent rise of PGF<sub>2α</sub> production; this PG induces (via paracrine, autocrine, and/or intracrine mechanism) an increase in NOS activity (Boiti *et al.* 2003); finally NO down-regulates progesterone levels (Boiti *et al.* 2000).

However, though the present data throw new light on the knowledge of the physiological mechanisms regulating luteal activity, further studies are needed to better understand the GNRH/GNRHR system fine-tuning that controls rabbit CL life span, including the presence of type II GNRHR or the involvement of PGF<sub>2α</sub> synthase and PGE<sub>2</sub>-9-ketoreductase, key enzymes of the PGF<sub>2α</sub> synthesis.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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